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Internal Relative Potency Factors for the Risk Assessment of Mixtures of Per- and Polyfluoroalkyl Substances (PFAS) in Human Biomonitoring

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Background: In human biomonitoring, blood is often used as a matrix to measure exposure to per- and polyfluoroalkyl substances (PFAS). Because the toxicokinetics of a substance (determining the steady-state blood concentration) may affect the toxic potency, the difference in toxicokinetics among PFAS has to be accounted for when blood concentrations are used in mixture risk assessment.

Objectives: This research focuses on deriving relative potency factors (RPFs) at the blood serum level. These RPFs can be applied to PFAS concentrations in human blood, thereby facilitating mixture risk assessment with primary input from human biomonitoring studies.

Methods: Toxicokinetic models are generated for 10 PFAS to estimate the internal exposure in the male rat at the blood serum level over time. By applying dose–response modeling, these internal exposures are used to derive quantitative internal RPFs based on liver effects.

Results: Internal RPFs were successfully obtained for nine PFAS. Perfluorobutanoic acid (PFBA), perfluorohexanoic acid (PFHxA), perfluorononanoic acid (PFNA), perfluorododecanoic acid (PFDoDA), perfluorooctane sulfonic acid (PFOS), and hexafluoropropylene oxide-dimer acid (HFPO-DA, or GenX) were found to be more potent than perfluorooctanoic acid (PFOA) at the blood serum level in terms of relative liver weight increase, whereas perfluorobutane sulfonic acid (PFBS) and perfluorohexane sulfonic acid (PFHxS) were found to be less potent. The practical implementation of these internal RPFs is illustrated using the National Health and Nutrition Examination Survey (NHANES) biomonitoring data of 2017–2018.

Discussion: It is recommended to assess the health risk resulting from exposure to PFAS as combined, aggregate exposure to the extent feasible. https://doi.org/10.1289/EHP10009

Introduction

As early as 1968, an unusually high nonionic fluorine fraction was noticed in human plasma.[1] After subsequent isolation of an organic fluoride substance, traced down to resemble the structure of perfluorooctanoic acid (PFOA), researchers suggested that exposure to commercial products would be the cause of widespread contamination of human tissue by organic fluorocompounds from anthropomorphic origin.[2] Years thereafter, human biomonitoring studies revealed high exposures to PFOA and perfluorooctane sulfonic acid (PFOS) in workers,[3–5] and PFOA, PFOS, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PDUnDA), perfluorohexane sulfonic acid (PFHxS), *N*-methylperfluorooctanesulfonamidoacetic acid (MeFOSAA), and *N*-ethylperfluorooctanesulfonamidoacetic acid (EtFOSAA) in communities.[6–10] Due to increasing awareness regarding the persistent, bioaccumulative, and toxic properties of perfluoroalkyl acids (PFAA),[11,12] regulatory measures have been put in place and are under development to mitigate exposure to these chemicals at the European level [e.g., under the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) Regulation and the Persistent Organic Pollutants (POPs) Regulation] and at the Mondial level (by inclusion in the annexes of the Stockholm and Rotterdam Conventions).[13] However, to date, exposure to legacy perfluoroalkyl carboxylic acids (PFCA) and perfluoroalkyl sulfonic acids (PFSA) is still apparent.[14–27]

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Due to the high bioaccumulation potential of legacy per- and polyfluoroalkyl substances (PFAS), they were substituted by substances with shorter carbon-chain length [e.g., perfluorobutanoic acid (PFBA), perfluorohexanoic acid (PFHxA), perfluorobutane sulfonic acid (PFBS), 6:2 fluorotelomer carboxylic acid (6:2 FTSA), 6:2 fluorotelomer carboxylic acid (6:2 FTCA)] and substances with other functional groups, like perfluoroether sulfonic and carboxylic acids [e.g., hexafluoropropylene oxide-dimer acid (HFPO-DA), hexafluoropropylene oxide-trimer acid (HFPO-TA), 6:2 chlorinated polyfluoroalkyl ether sulfonic acid (6:2 Cl-PFESA), ammonium 4,8-dioxa-3H-perfluorononanoate (ADONA), and Nafion by-product 2 (BP2)].[28–30] These substances are presumed to be of less concern in comparison with their predecessors because of the more rapid elimination from the blood in experimental animal species.[31] However, whereas elimination half-lives vary from hours to several days in the rat, mouse, and monkey, in humans it may take one to several months before a reduction of 50% of chemicals such as ADONA,[32] PFBS,[33,34] PFHxA,[34–36] perfluoroheptanoic acid (PFHpA),[34,36] or perfluoropentane sulfonic acid (PFPeS)[34] in the blood is reached, leaving to question whether these alternatives truly are of no concern relative to their accumulation in humans, wildlife, and livestock.

The levels of PFBA, PFHxA, and PFBS in blood of the general population in Europe are nonetheless relatively low[14,18–21,24,27]; however, ADONA and HFPO-DA have not been screened for on a regular basis. Quantifiable plasma concentrations were reported in two targeted monitoring studies in young men in Germany (ADONA) and in employees of a perfluoropolymer production plant in the Netherlands (HFPO-DA),[37–39] but two other studies did not find any evidence for exposure to ADONA.[25,27] Studies in the United States (U.S.) revealed no quantifiable serum levels for HFPO-DA and no or very low HFPO-DA urine levels in the general population and in residents living in a hot-spot region.[16,40,41] Exposure to BP2, perfluoro-3,5,7,9-tetraoxadecanoic acid (PFO4DA), HFPO-TA, and 6:2 Cl-PFESA was, however, recently discovered to be prominent in populations in Asia[42,43] and the U.S.[41]

Members of the groups of PFCA and PFSA have been associated with hepatotoxicity, nephrotoxicity, hemotoxicity, reproductive toxicity, developmental toxicity, immunotoxicity, endocrine disruption (thyroid), and carcinogenicity in toxicological experimental animal

models (in rats, mice, or monkeys), which correlate fairly well with findings in human epidemiological studies.[44,45] The perfluoroether carboxylic acids ADONA and HFPO-DA illustrate a toxicity profile that is comparable to the PFCA and PFSA in experimental animal studies.[46–49] However, for many of the emerging PFAS, little[50–54] to no toxicity information is available, urging regulatory bodies to come up with advanced and novel high-throughput testing strategies to facilitate risk assessment or to focus on concern-driven exposure mitigation for PFAS as a group.[13,55,56] Still, even for the PFAS for which considerable experimental data on toxicokinetics and toxicity are present, risk assessment proves challenging. For instance, the European Food Safety Authority (EFSA) could only establish a sum tolerable weekly intake (TWI) for four PFAS (PFOA, PFNA, PFHxS, PFOS) after it was asked by the European Commission to prepare a scientific opinion on the risks to human health related to the presence of PFAS in food.[45]

The aggregate exposure to PFAS stems from a large variety of different sources, such as diet (including drinking water),[45,57–59] consumer products,[60,61] and occupational sources,[60] with diet being the predominant exposure pathway.[45,62] Human biomonitoring is of added value in risk assessment because, apart from its importance as an early warning signal, it serves as an empirical measure for aggregate exposure.[63] In experimental animal species, PFAA are readily absorbed from the gastrointestinal tract and are excreted unmetabolized.[64–68] They deposit primarily in the blood, liver, and kidney,[66,67,69–74] although for some of the substances (e.g., PFBA, HFPO-DA) partitioning into organs and tissues other than the liver and adipose tissue (e.g., kidney, spleen, brain, lung) remains largely unknown due to lack of data availability. The limited human data available for PFOA and PFOS on serum-to-liver partitioning suggest a substantially higher distribution of both compounds to the liver in comparison with distributions for rodents at comparable blood concentrations.[75] Due to the deposition mainly in blood, liver, and kidney, blood is considered to be the most suitable matrix to evaluate exposure to these substances in human biomonitoring in comparison with matrices such as urine, hair, saliva, or exhaled air.[76]

Previously, we presented relative potency factors (RPFs) for a set of 23 PFAS to assess the cumulative risk on oral (external) exposure.[77] This set of RPFs is useful when estimating the risk resulting from mixtures of PFAS in, for instance, drinking water or food samples. The methodology is based on the principle of dose addition, which assumes that chemicals contribute to a common adverse effect and behave as if they were a dilution of one another.[78] Exposure of each substance is converted to equivalents of an index compound (PFOA in this case) by multiplying with an RPF. Subsequently, these equivalents are summed to provide the cumulative exposure. All further calculations can then be performed as equivalents of the index compound.

In human health risk assessment of persistent, bioaccumulating substances, it is important to consider body burdens or serum concentrations across time rather than external intake doses.[75] We propose that specific, internal RPFs should be used when assessing the risk of exposure to multiple PFAS at the blood (serum or plasma) level. That is, kinetics determining the internal dose after (external) intake differ between PFAS, and therefore potencies based on external doses will not be the same as potencies based on internal doses. For example, based on external doses HFPO-DA has been shown to be less potent than PFOA in the male rat when looking at relative liver weight increase[77,79] but was regarded to be more potent when these external concentrations were converted to blood serum concentrations.[79] Considering the fact that HFPO-DA shows a lower degree of accumulation in male rats in comparison with PFOA,[80,81] the differences in external potency between HFPO-DA and PFOA may at least partly be explained by differences in toxicokinetics. This topic will be further addressed in the "Discussion" section.

In the current study, we generated a toxicokinetic model for 10 PFAS to estimate the internal exposure in the male rat at the blood serum level over time. Subsequently, these internal exposures were used to derive internal RPFs for nine PFAS (including the index compound) based on relative liver weight increase in the male rat. We close by illustrating the use of these internal RPFs, having combined them with human biomonitoring data from the National Health and Nutrition Examination Survey (NHANES) 2017–2018 cycle.[82]

Materials and Methods

Conceptual Overview

Derivation of internal RPFs required various steps (Figure 1; steps 1–6). First, a toxicity database was compiled, which provided information on a common toxic adverse effect, whereby other factors influencing relative potency estimates were eliminated as much as possible (e.g., difference in exposure duration, species, sex, strain) (step 1). Subsequently, for each PFAS, a toxicokinetic model was obtained (step 2) and implemented (step 3). Correct implementation was confirmed by comparing the modeled serum concentration-time curves with the experimentally derived serum concentrations of single-dose studies. After that, the modeled serum concentration-time curves were verified against experimentally derived serum concentrations in repeated-dose studies (step 4). Then, implemented models were used to convert the external administered doses of the studies in the toxicity database into internal doses by modeling the exposure scenarios of the experimental animal studies (step 5). Finally, dose–response curves were fitted to the liver weight responses plotted

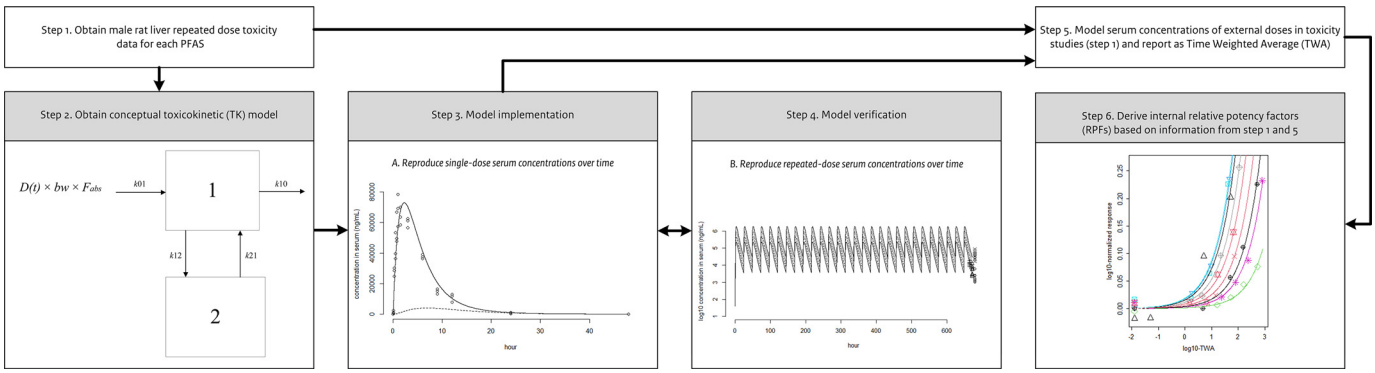

Figure 1. Conceptual overview of the methods applied to derive the internal relative potency factors (RPFs).

against the internal doses (obtained in step 1) to be able to derive RPFs (step 6).

Gathering Toxicity Data (Step 1)

PFAS have been associated with disrupted hepatic metabolism, lipid accumulation in the liver, and adverse outcomes such as steatosis, necrosis, and hepatocellular tumor formation.[77,83,84] Although one study suggests that some of these effects of PFAS on the liver involve the activation of the peroxisome proliferator-activated receptor-alpha (PPARα) with uncertain relevance to human health,[85] other studies suggest several other nuclear receptors [e.g., peroxisome proliferator–activated receptor γ (PPARγ), constitutive androstane receptor (CAR)] and cytotoxicity may play a role in adverse effects on the liver,[77] leading to the conclusion that the mechanism(s) underlying these adverse effects are still unclear and deserve further investigation.[83] We consider the PFAS-induced hepatotoxic effects in rodents, including hypertrophy and liver weight increase, relevant for risk assessment and, thus, suitable for derivation of RPFs.[77]

A database of liver end points was previously created for 16 PFAS based on information retrieved from public literature.[77] To ensure that the differences in potency would not be affected by differences in experimental setup, data were obtained from studies with the same species (rat), sex (male), and exposure route (oral) and comparable exposure duration (42–98 d).[86–94] PFAS have mostly been studied under a subchronic exposure regimen in rats (in comparison with, for instance, mice) and therefore selection of this study protocol with rats resulted in the highest number of PFAS with toxicity data available. Chronic studies are available only for a limited number of PFAS (e.g., PFHxA,[95] HFPO-DA,[96] PFOS,[97] PFOA[98]), and although there are also subacute studies available for quite a number of PFAS (for a recent overview see the studies cited in EFSA[45] and ATSDR[44]), they are of lower preference due to the shorter exposure duration in comparison with subchronic studies. In general, male rats appear more sensitive to PFAS-induced liver toxicity in comparison with females and show more severe liver pathology in comparison with females at equipotent doses.[94,97,99,100] These findings support the selection of male rat toxicity data for derivation of RPFs. The database may be found in Table S1.

In Bil et al.,[77] we illustrated that derivation of oral external RPFs based on liver hypertrophy, absolute liver weight increase, and relative liver weight increase resulted in similar RPFs for absolute and relative liver weight and somewhat smaller RPFs for hepatic hypertrophy. Because the database for relative liver weight was most complete, the final external RPFs were based on this end point. For the same reason, relative liver weight was selected as the end point to derive internal RPFs. In addition, this allowed for direct comparison of internal and external RPFs.

Toxicokinetic Models and Equations (Step 2)

The public literature was screened for toxicokinetic data and models for the 16 PFAS included in the toxicity database, using search engine Google Scholar as well as screening the literature cited in several scientific reports and publications.[44,45,79] For 10 PFAS [PFBA,[68] PFHxA,[81] PFOA,[81] PFNA,[101] PFDA,[81] perfluorododecanoic acid (PFDoDA),[73] PFBS,[102] PFHxS,[102] PFOS,[102] HFPO-DA[80]], sufficient model parameter values were obtained from single-dose experiments. In most studies, multiple doses and exposure routes were tested. For concordance with the toxicity database, toxicokinetic studies with male rats exposed via the oral route and treated with a dose closest to the doses used in the oral repeated-dose toxicity studies, were selected.

We implemented a compartmental model that incorporated mono- and biphasic processes (Figure 2). Compartments 1 and 2 represent the tissues with relatively fast and slow partitioning, respectively. The corresponding differential equations are:

$$\frac{dA}{dt} = D(t) \times bw \times F_{abs} - A \times k01, \quad (1)$$

$$\frac{dC1}{dt} = \frac{A \times k01}{V1} - k10 \times C1 - k12 \times C1 + k21 \times C2, \quad (2)$$

$$\frac{dC2}{dt} = k12 \times C1 - k21 \times C2, \quad (3)$$

where $D(t) =$ bolus dose of a PFAS (milligrams per kilogram body weight) at time point t (hours), $bw =$ body weight (kilograms), $A =$ the fraction (F_{abs}) of the amount of a PFAS in the gastrointestinal tract that will be absorbed over time (nanograms), $k01 =$ the absorption rate constant from the gastrointestinal tract to the central compartment (Compartment 1) (1/unit time), $C1$ and $C2 =$ PFAS concentration in Compartment 1 and 2 (nanograms per milliliter), $V1 =$ the volume of distribution of Compartment 1 (milliliters), $k10 =$ the elimination rate constant from Compartment 1 (1/unit time), and $k12$ and $k21 =$ the transfer rate constants between compartments 1 and 2 (1/unit time).

Model Implementation (Step 3)

To implement a kinetic model for each PFAS, values of the parameters mentioned in Equations 1, 2, and 3 were required. The publications on the single exposure toxicokinetic experiments provided most parameter values, i.e., on F_{abs}, T_{max}, $V1$, $k10$ elimination half-life (in case of monophasic kinetics) and alpha and beta elimination half-lives (in case of biphasic kinetics), from which the other required parameters ($k10$, $k01$, $k12$, $k21$) were obtained using Equations 4–6 below.

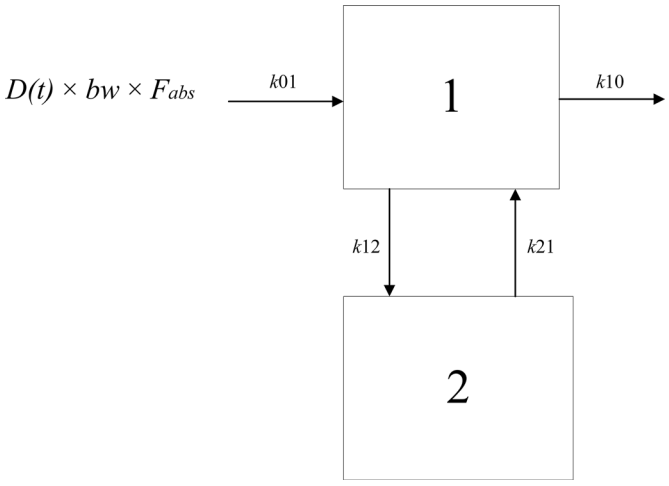

Figure 2. Schematic overview of the applied compartment model with first-order input (oral exposure). The amount $A(t)$ of a PFAS (per- and polyfluoroalkyl substances) enters the gastrointestinal tract as the product of the externally administered dose $D(t)$ and body weight (bw), after which a fraction (F_{abs}) of the administered amount is absorbed (with rate constant $k01$) into the central compartment (Compartment 1). Elimination from the central compartment is characterized by the rate constant $k10$. Parameters $k12$ and $k21$ are transfer rate constants between Compartments 1 (central compartment) and 2 (peripheral compartment), which both are set to zero in case the kinetics of a PFAS are monophasic. Note: PFAS, per- and polyfluoroalkyl substances.

The elimination rate ($k10$) was calculated from the $k10$ elimination half-life[103] using Equation 4:

$$k10 = \left(\frac{\ln(2)}{k10\,T1/2} \right), \tag{4}$$

where $k10$ $T1/2$ is the known $k10$ elimination half-life.

The absorption rate ($k01$) was calculated from the T_{max}[103,104] using Equation 5:

$$T_{max} = \frac{\ln\left(\frac{k01}{k}\right)}{k01 - k}, \tag{5}$$

where k is either the known elimination rate ($k10$) in case of a one-compartment model or the known alpha rate (from Equation 4, where $k10$ $T1/2$ is substituted by α $T1/2$) in case of a two-compartment model. This equation was solved numerically by varying $k01$.

Obtaining the absorption and elimination rates was done by using the relatively simple equations (Equations 4 and 5) above. The transfer rates between Compartment 1 and Compartment 2 ($k12$ and $k21$) were derived from the alpha rate, beta rate, and $k10$ by obtaining the roots of a more complex quadratic equation. The solution of this equation, and how $k21$ and $k12$ are derived from this, is reported in Supplemental Material, "Toxicokinetic model parameterization."

Correct implementation of the compartment models was confirmed by visual inspection of the modeled serum concentration plotted together with the empirically obtained serum concentrations over time,[68,73,81,101,102,105] and by evaluating the ratio between the modeled and reported area under the curve (AUC), maximum serum concentration (C_{max}), and time at C_{max} (T_{max}) values.

The original serum concentration data in Dzierlenga et al.[81] and Huang et al.[102] were retrieved from the Chemical in Effects Biological Systems (CEBS) database (https://manticore.niehs.nih.gov/cebssearch), and the study report of Gannon[105] was obtained from the Health and Environmental Research Online (HERO) database (https://hero.epa.gov/). The data presented in the other publications were digitalized using DigitizeIt software (version 2.3.3; https://www.digitizeit.xyz/).

Verification (Step 4)

For seven PFAS (PFHxA, PFOA, PFNA, PFDA, PFBS, PFHxS, PFOS), measured serum concentrations were available to verify the performance of the models to simulate the serum concentrations after repeated dosing. Verification was done by plotting modeled serum concentrations together with empirically obtained repeated-dose serum concentrations obtained after daily exposure for 28 d.[99,100] These serum concentration data were retrieved from the CEBS database as well. Furthermore, the ratio between the modeled and reported mean serum concentration at the end of the study was evaluated.

Calculation of Internal Doses in the Serum for the Repeated-Dose Toxicity Studies (Step 5)

The exposure conditions of the experimental animal toxicity studies reported in Table S1 were implemented in the congener-specific kinetic models to convert the external administered doses under these conditions to internal doses, represented as time-weighted average (TWA) serum concentration in milligrams per milliliter. When available, the ratio between the modeled and reported mean serum concentration at the end of the study was also evaluated to verify the model predictions.

Because the maximum increase in relative liver weight by PFOA and PFOS is observed already after short-term exposure and does not change after longer exposure durations,[88,91,106] the different subchronic exposure durations of the available studies were only considered to have a marginal impact on the liver response. For this reason, dose metrics depending strongly on the exposure duration, such as the AUC (in milligrams multiplied by hours per milliliter), were deemed unsuitable.

Dose–Response Analysis and Derivation of Internal RPFs (Step 6)

The RPF approach requires that *a*) chemicals contribute to a common effect, *b*) their dose–response curves are (approximately) parallel on log–dose scale, and *c*) chemicals do not interact.[107] Parallelism ensures a constant dose factor between the curves at any effect level; thus the RPFs do not depend on the benchmark response, in case of continuous data such as relative liver weight.

Dose–response modeling and derivation of internal RPFs were performed in a manner similar to the procedure set out in Bil et al.[77] In short, dose–response modeling was performed using PROAST software (version 70.2; https://www.rivm.nl/en/proast). A four-parameter exponential model,

$$y = a\left(c^{1 - e^{\left(-\left(\frac{x}{b}\right)^d\right)}} \right), \tag{6}$$

was fitted to the (continuous) relative liver weight data plotted against the TWA obtained at step 5.

In a covariate analysis, parallel curves were fit to the data by applying the same shape parameters (maximum response parameter c and the steepness parameter d) in the four-parameter exponential model to all PFAS, but allowing the background (parameter a), the potency (parameter b), and the residual variance to be different between PFAS.[108,109] A good description of the data of each PFAS by parallel curves was confirmed by visual inspection.

The RPF is defined as the ratio of two (equipotent) doses, x_{PFOA} for the index compound and x_i for PFAS i, which both result in the same relative change in relative liver weight response (y):

$$RPF_i = \frac{x_{PFOA}}{x_i}, \tag{7}$$

When assuring the curves of the two PFAS are parallel, x/b in the exponential model (Equation 6) has the same value for both PFAS:

$$\frac{x_{PFOA}}{b_{PFOA}} = \frac{x_i}{b_i}, \tag{8}$$

Combining Equations 7 and 8 gives Equation 9,

$$RPF_i = \frac{b_{PFOA}}{b_i}, \tag{9}$$

which shows that the RPF of each PFAS can be directly obtained from its potency parameter b and that of PFOA. Calculation of the RPFs and their 90% confidence intervals (CIs) based on potency parameter b was performed using PROAST software.

Calculating PFAS Mixture Exposure: Practical Implementation of Internal RPFs

The human biomonitoring data of NHANES[82] were used to illustrate how to use internal RPFs in combination with a human biomonitoring data set. NHANES is designed to gather representative

information for the U.S. general population. The National Center for Health Statistics (NCHS) Research Ethics Review Board reviewed and approved the study protocol. To participate in the survey, all respondents gave informed written consent, parents or guardians provided written permission for participants younger than 18 y, and children 12–17 y old provided assent.

In the 2017–2018 cycle, PFAS were measured in a random subsample of 1,929 individuals ages 12 and older. Characteristics of this subpopulation can be found on the NHANES website.[82] Blood serum of each individual was screened for the quantitative detection of Me-FOSAA, PFHxA, PFOA (both linear and branched isomers), PFNA, PFDA, PFUnDA, PFHxS, PFHpS, PFOS (both linear and branched isomers), 6:2 Cl-PFESA, ADONA, and HFPO-DA. Lower limit of detection (LOD) for all substances was 0.100 nanograms per milliliter (ng/mL). Additional information on the analytical methods, substance full names, and CAS numbers, and an overview of the data can be found in Table S2 and Figure S1, respectively.

To obtain PFOA equivalents (PEQs), PFAS serum concentrations of each participant were multiplied by the internal RPFs when both serum concentration data and internal RPFs were available. PEQs were summed to calculate the cumulative PFAS exposure per individual. NCHS recommends to use weights to account for unequal selection probabilities caused by the clustered design of NHANES and to account for oversampling certain demographic groups, if the sample would have to represent the U.S. general population.[110] Because our goal was not to interpret the risk of the U.S. general population but rather to illustrate how to use internal RPFs in combination with a human biomonitoring data set, weighing was considered out of the scope of this paper.

Software

All calculations were performed in R (version 4.0.0, R Core Team). In addition, R packages were used for the kinetic models: deSolve (version 1.32),[111] for dose–response analysis and deriving RPFs: PROAST software (version 70.2, RIVM, https://www.rivm.nl/en/proast), and for downloading and analyzing the NHANES data: nhanesA (version 0.6.5.3, Endres, https://CRAN.R-project.org/package=nhanesA).

Results

Model Parametrization (Step 2)

According to the authors of the original studies, the measured serum concentrations could be effectively described by either monophasic or biphasic kinetics.[68,73,80,81,101,102] These toxicokinetic evaluations were implemented in our analysis (see Supplemental Materials, "Toxicokinetic model parameterization;" Tables S3–S4; Figures S2–S8). Only PFNA was regarded to be better described by monophasic kinetics rather than biphasic kinetics.

The starting parameters F_{abs}, T_{max}, $V1$, $k10$ $T1/2$, α $T1/2$, and β $T1/2$ were used to obtain the other required parameters $k10$, $k01$, $k12$, $k21$ by using Equations 4 and 5, and Equations S7–S11 (Supplemental Material, "Toxicokinetic model parametrizations"). In some cases the $k10$ $T1/2$ (HFPO-DA), $V1$ (PFDoDA), or $k10$ $T1/2$ and $V1$ (PFNA) were not provided, and thus these values were obtained by optimizing the toxicokinetic models to the empirically obtained serum concentrations over time.[73,101,105] Further details are provided in Supplemental Material, "Toxicokinetic model parameterization."

The toxicokinetic model parameters listed in Table 1 were subsequently used to implement the toxicokinetic compartment models for PFBA, PFHxA, PFOA, PFNA, PFDA, PFDoDA, PFBS, PFHxS, PFOS, and HFPO-DA (Equations 1–3). When the kinetics of a PFAS could be explained by monophasic kinetics, the transfer rates $k12$ and $k21$ were set to zero.

Model Implementation and Verification (Step 3 and 4)

For each model, the single-dose serum curve was plotted together with the original serum concentration data (Figures S9–S18). As plasma measures for HFPO-DA were performed, a 1:1 serum to plasma ratio was assumed according to the observations in Ehresman et al.[112] Additionally, verification for repeated dose exposure was

Table 1. Toxicokinetic model parameter values of per- and polyfluoroalkyl substances (PFAS).

Compound	Study	Evaluation of TK model fit	Dose (mg/kg)	F_{abs} (−)	T_{max} (h)	$V1$ (mL/kg)	α $T1/2$ (h)	β $T1/2$ (h)	$k10$ $T1/2$ (h)	$k01$ (1/h)	$k10$ (1/h)	$k12$ (1/h)[i]	$k21$ (1/h)[i]
PFBA	Chang et al.[68]	Monophasic[a]	30	1.0[b]	1.25[a]	209[a]	—	—	9.22[a]	3.0[a]	0.075[h]	—	—
PFHxA	Dzierlenga et al.[81]	Biphasic[a]	160	1.0[b]	0.890[a]	348[a]	1.46[a]	13.7[a]	1.63[a]	2.2[f]	0.43[h]	0.039	0.056
PFOA	Dzierlenga et al.[81]	Monophasic[a]	12	1.0[b]	6.37[a]	154[a]	—	—	258[a]	0.92[f]	0.0027[h]	—	—
PFNA	Tatum-Gibbs et al.[101]	Monophasic[c]	3	1.0[b]	—	170[j]	—	—	—	1.0[b]	0.00025[j]	—	—
PFDA	Dzierlenga et al.[81]	Biphasic[a]	10	1.0[b]	9.06[a]	228[a]	123[a]	995[a]	478[a]	0.50[f]	0.0015[i]	0.0021	0.0027
PFDoDA	Kawabata et al.[73]	Monophasic[a]	50	1.0[a]	120[a,g]	663[d]	—	—	1,327[a,g]	0.036[f]	0.00052[h]	—	—
PFBS	Huang et al.[102]	Biphasic[a]	20	1.0[b]	2.18[a]	148[a]	2.37[a]	5.36[a]	2.73[a]	0.68[f]	0.25[h]	0.018	0.15
PFHxS	Huang et al.[102]	Monophasic[a]	16	1.0[b]	5.89[a]	137[a]	—	—	396[a,g]	1.1[f]	0.0018[h]	—	—
PFOS	Huang et al.[102]	Biphasic[a]	2	1.0[b]	14.3[a]	280[a]	74.4[a,g]	972[a,g]	478[a,g]	0.24[f]	0.0015[h]	0.0040	0.0045
HFPO-DA	Gannon et al.[80,e]	Biphasic[a]	10	1.0[a]	—	142[a]	2.8[a]	72.2[a]	—	3.3[a]	0.24[k]	0.0099	0.010

Note: —, no data; α $T1/2$, alpha elimination half-life; β $T1/2$, beta elimination half-life; HFPO-DA, hexafluoropropylene oxide-dimer acid; F_{abs}, fraction of the amount of a PFAS in the gastrointestinal tract that will be absorbed over time; $k01$, absorption rate constant from the gastrointestinal tract into Compartment 1; $k10$, elimination rate constant from Compartment 1; $k10$ $T1/2$, elimination half-life from Compartment 1; $k12$, transfer constant from Compartment 1 to Compartment 2; $k21$, transfer constant from Compartment 2 to Compartment 1; PFAS, per- and polyfluoroalkyl substances; PFBA, perfluorobutanoic acid; PFBS, perfluorobutane sulfonic acid; PFDoDA, perfluorododecanoic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexane sulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid; TK, toxicokinetic; T_{max}, time at maximum serum concentration; $V1$, volume of distribution of Compartment 1.

[a]Value provided in the study from oral, single exposure of males at the dose provided in column "dose."

[b]Based on assumption in the original studies.

[c]In the original publication the authors report that the data illustrated biphasic kinetics. See Supplementary Material, "Toxicokinetic model parameterization."

[d]Calculated in this study. See Supplemental Material, "Toxicokinetic model parameterization."

[e]Serum concentrations per individual animal over time reported in Gannon.[105] Note that 12-h concentrations were assumed to be transposed in the original report. See also Supplementary Material, "Toxicokinetic model parameterization."

[f]Calculated according to Equation 5.

[g]Provided in study in different unit (days).

[h]Calculated according Equation 4.

[i]Calculated according to method in Supplemental Material, "Toxicokinetic model parameterization."

[j]Optimized; note that study reports other values for $V1$ and $k10$. See remarks in Supplemental Material, "Toxicokinetic model parameterization."

[k]Optimized according to the method shown in Supplemental Material, "Toxicokinetic model parameterization."

Table 2. Comparison of reported and modeled area under the curve (AUC), maximum serum concentration (C_{max}), and time at maximum serum concentration (T_{max}) for the single-dose experiments.

		AUC				C_{max}				T_{max} (h)		
Compound	Study	Reported	Modeled	Ratio	Unit[b]	Reported	Modeled	Ratio	Unit[b]	Reported	Modeled	Ratio
PFBA	Chang et al.[68]	1,900	1,600	1.2	μg × h/mL	130	130	1.0	μg/mL	1.3	1.3	1.0
PFHxA	Dzierlenga et al.[81]	3.5	3.4	1.0	mM × h	0.97	0.96	1.0	mM	0.89	0.91	1.0
PFOA	Dzierlenga et al.[81]	70	67	1.0	mM × h	0.19	0.18	1.1	mM	6.4	6.4	1.0
PFNA	Tatum-Gibbs et al.[101]	15,000	18,000	0.8	mg × h/L	22	18	1.2	μg/mL	—	8.8	—
PFDA	Dzierlenga et al.[81]	59	54	1.1	mM × h	0.083	0.082	1.0	mM	9.1	11	0.8
PFDoDA	Kawabata et al.[73]	—	140	—	mM × h	54	70	0.8	μg/mL	120	120	1.0
PFBS	Huang et al.[102]	1.8[a]	1.8	1.0	mM × h	0.25	0.24	1.0	mM	2.2	2.3	1.0
PFHxS	Huang et al.[102]	170[a]	150	1.1	mM × h	0.29	0.29	1.0	mM	5.9	5.9	1.0
PFOS	Huang et al.[102]	9.9[a]	8.9	1.1	mM × h	0.010	0.013	0.8	mM	14	17	0.8
HFPO-DA	Gannon et al.[80]	—	0.88	—	mM × h	—	0.17	—	mM	—	0.86	—

Note: —, no data; HFPO-DA, hexafluoropropylene oxide-dimer acid; PFBA, perfluorobutanoic acid; PFBS, perfluorobutane sulfonic acid; PFDoDA, perfluorododecanoic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexane sulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid.
[a]AUC units in Huang et al.[102] should be mM × h (C.R. Blystone, personal communication).
[b]Units reported in the study are used.

performed for seven PFAS (PFHxA, PFOA, PFNA, PFDA, PFBS, PFHxS, PFOS) (Figures S19–S27). We show the study selection and model results for PFBS below for illustrative purposes.

Single-dose modeling verification. Because the repeated-dose study for PFBS in the toxicity database was dosed at 0, 30, 100, 300, and 1,000 mg/kg body weight/d, the 20 mg/kg dose was selected from the toxicokinetic experiments in Huang et al.[102] (dosed at 4, 20, and 100 mg/kg) because this dose was in the same range as the doses of the repeated-dose study. The same line of reasoning was used to select the doses for the other substances. To verify correct implementation, the single-dose experiments informing the parameter values were modeled. Table 2 shows the modeled AUC, C_{max}, and T_{max} in comparison with the values for these parameters reported in the original studies. The ratios between modeled and reported values range from 0.8 to 1.2.

Figure 3 shows the modeled single-dose serum concentration of PFBS over time, plotted together with the reported mean serum concentrations from Huang et al.[102] Figures of the simulations of single-dose experiments for PFBA, PFHxA, PFOA, PFNA, PFDA, PFDoDA, PFHxS, PFOS, and HFPO-DA are included as Figures S9–S18.

Repeated-dose modeling verification. Figure 4 shows the modeled repeated-dose serum concentration of PFBS over time, plotted together with the mean measured serum concentrations shown in NTP[99] at day 28. In Table 3 the modeled and reported serum concentrations are listed, showing that the model is able to predict the measured concentrations after 4 wk (= 672 h) of daily exposure, with ratios ranging from 0.5 to 5.5. Figures S19–S27 show the simulation of 28-d repeated-dose experiments for PFHxA, PFOA, PFNA, PFDA, PFHxS, and PFOS, in addition to that of PFBS.

Calculation of Internal Doses in the Serum for the Repeated-Dose Toxicity Studies (Step 5)

The internal doses were calculated for each of the doses in the repeated-dose toxicity studies as presented in Table S1. Please note that no subchronic toxicity study for PFDA was available, hampering further calculation of an internal RPF for this substance with the toxicokinetic model available. Modeled TWA and serum concentration at the end of the study are provided for each substance in Table 4, together with the serum concentration measured at the end of the dosing experiments, when available. The ratios between modeled and reported serum concentrations at the end of the study ranged from 0.2 to 7.0. The modeled TWA serum concentration was used as dose metric for dose–response modeling and RPF derivation.

Dose–Response Analysis and Derivation of Internal RPFs (Step 6)

Dose–response curves were fit to the data using an exponential model with compound as a covariate on the background parameter (a), the potency parameter (b), and the residual variance (Equation 6). In this analysis the parameters c and d, determining the shape of the curves, were the same for each PFAS. This approach resulted in dose–response curves which have a study-specific background (Figure 5, left panel) and potency (Figure 5, right panel) but have

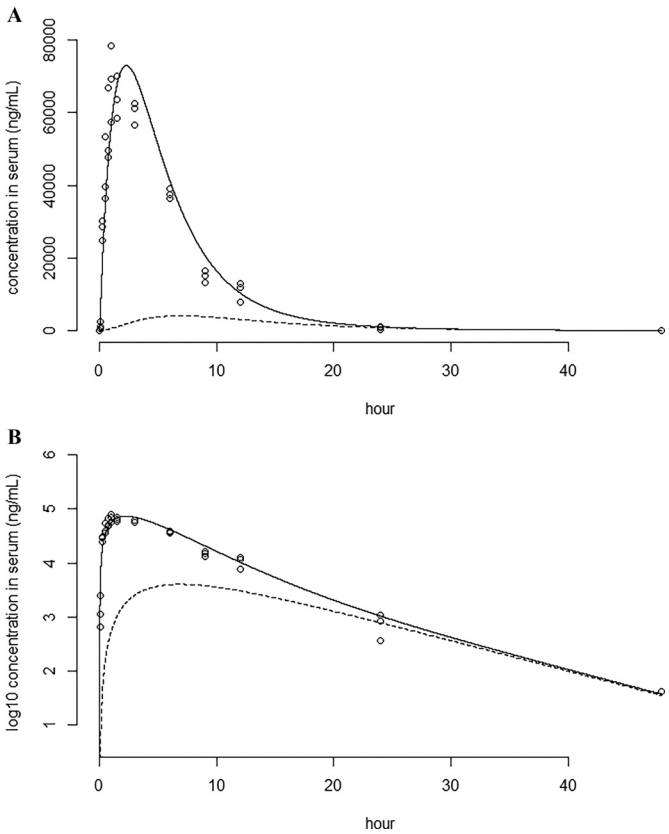

Figure 3. Perfluorobutane sulfonic acid (PFBS) serum concentration plotted against time (h) after an oral single dose of 20 mg/kg in male rats. Note: The solid line is the modeled concentration in the serum (nanograms per milliliter). The dashed line indicates the concentration in the peripheral compartment (nanograms per milliliter). Circles are the measured concentrations of the individual animals ($n = 3$) retrieved from Huang et al.[102] Figures are plotted on a linear (A) and $\log_{10}$ (B) scale.

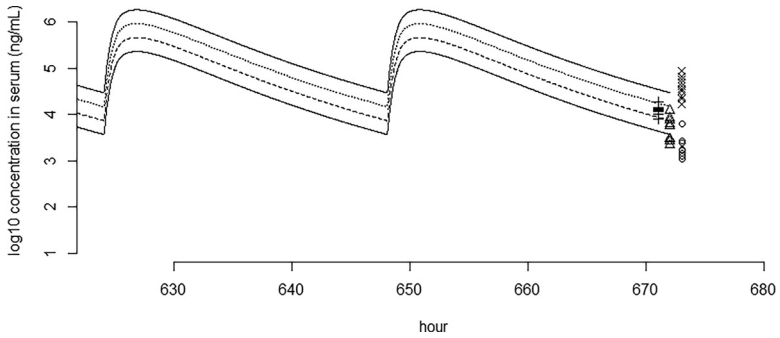

Figure 4. Modeled perfluorobutane sulfonic acid (PFBS) serum concentrations over time upon daily dosing together with 28-d serum concentrations (symbols) in male rats ($n = 10$) as reported in NTP,[99] plotted on $\log_{10}$-scale. Only the last ~48 h of the experiment are shown. Note: Lowest solid line and circles = 62.5 mg/kg body weight/d; dashed line and triangles = 125 mg/kg body weight/d; dotted line and plusses = 250 mg/kg body weight/d; upper solid line and crosses = 500 mg/kg body weight/d. To distinguish measured points, they have been shifted slightly.

the same shape—i.e., they are parallel on the log-dose scale. In Figure 6 the curves from Figure 5 are plotted separately to confirm that curves with the same shape provide a good description of the measured data of all PFAS. Only one data point for HFPO-DA could not be described by the model, because the confidence interval was slightly next to the dose–response curve. However, this deviation was considered marginal, and HFPO-DA was included for further derivation of the RPFs.

Table 3. External dose and modeled and reported concentrations at the end of the study for each of the doses in 28-d repeated-dose toxicity studies for male rats specifically.

			Concentration at end of the study (mg/mL)		
Compound	Reference	External dose (mg/kg body weight/d)	Modeled	Reported mean	Ratio
PFBS	NTP[99]	62.6	3.7	2.2	1.3
		125	7.4	5.3	1.4
		250	15	12	1.3
		500	30	43	0.7
PFHxS	NTP[99]	0.625	71	66	1.1
		1.25	140	92	1.5
		2.5	280	130	2.2
		5	570	160	3.6
		10	1,100	200	5.5
PFOS	NTP[99]	0.312	13	24	0.5
		0.625	26	52	0.5
		1.25	52	94	0.6
		2.5	100	170	0.6
		5	210	320	0.7
PFHxA	NTP[100]	62.6	1	0.38	2.6
		125	2	0.50	4.0
		250	4	1.3	3.1
		500	8.1	3.4	2.4
		1,000	16	11	1.5
PFOA	NTP[100]	0.625	48	51	0.9
		1.25	96	73	1.3
		2.5	190	95	2.0
		5	380	110	3.5
		10	770	150	5.1
PFNA	NTP[100]	0.625	95	57	1.7
		1.25	190	160	1.2
		2.5	380	380	1.0
		5	760	360	2.1
PFDA	NTP[100]	0.156	9	8.5	1.1
		0.312	18	23	0.8
		0.625	36	43	0.8
		1.25	72	100	0.7
		2.5	140	260	0.5

Note: NTP, National Toxicology Program; PFBS, perfluorobutane sulfonic acid; PFDA, perfluorodecanoic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexane sulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid.

Internal RPFs

The RPF analysis indicates that, based on TWA blood concentrations, the range in potencies relative to PFOA varies between 0.2 and 10 (Table 5). PFBA, PFHxA, PFNA, PFDoDA, PFOS, and HFPO-DA are more potent than PFOA, whereas PFBS and PFHxS are less potent in comparison with PFOA in terms of relative liver weight increase. Because dose–response modeling was used for RPF derivation, the uncertainty of the underlying toxicity data could be taken into account. The 90% CI ranges of the RPFs are illustrated in Figure 7 and are provided as numerical values in Table S5.

Calculating PFAS Mixture Exposure: Practical Implementation of Internal RPFs

NHANES 2017–2018 human biomonitoring measurements. In NHANES 2017–2018, several of the PFAS measured were detected in a large fraction of the population, with exception of PFHxA, ADONA, and HFPO-DA, which were detected above LOD in nine, zero, and one individuals, respectively. Median exposure values of PFOA (linear), PFNA, PFHxS, PFOS (linear and branched isomers) were highest, although exposure to PFDA, PFUnDA, PFHpS, Me-FOSAA, and 6:2 Cl-PFESA was also prominent in the sampled population. A summary box plot of the measured serum concentrations is provided in Figure S1.

Cumulative exposure of PFAS in the NHANES sample expressed as sum of PFOA equivalents (PEQs). Exposure to six PFAS (PFHxA, PFOA, PFNA, PFHxS, PFOS, HFPO-DA) for each participant in the NHANES study population was converted to PEQs by combining serum concentrations (above LOD) with internal RPFs. For the other PFAS (ADONA, PFDA, PFHpS, Me-FOSAA, and 6:2 Cl-PFESA), no internal RPFs are available, so these PFAS could not be included in the assessment of the cumulative PEQ exposure. A cumulative density plot of the sum PEQ in the NHANES study population is presented in Figure 8. The density plot may be found in Figure S28. The black line represents cumulative exposure as sum PEQ (nanograms per milliliter) of all PFAS from which internal RPFs were derived. Furthermore, for each individual the contribution of each PFAS to the individual's total PEQ concentration was derived. Exposure to PFOS contributes highest to the sum PEQ (Figure S29). Together, linear and branched PFOS contributed approximately 80% to the sum PEQ on average.

Discussion

In the current paper, we present a methodology based on internal RPFs, which allowed us to perform mixture risk assessment for PFAS based on serum concentration data. The compartment

Table 4. External dose, modeled TWA (time-weighted average) serum concentration, and modeled and reported concentrations at the end of the study for each of the doses in subchronic repeated-dose toxicity studies for male rats specifically.

				Concentration at end of the study (mg/mL)		
Substance	Reference	External dose (mg/kg body weight/d)	Modeled TWA (mg/mL)	Modeled	Reported mean	Ratio
PFBS	Lieder et al.[86]	30	0.016	0.0018	—	—
		100	0.053	0.0059	—	—
		300	0.16	0.018	—	—
		1,000	3.7	0.059	—	—
PFHxS	Butenhoff et al.[87]	0.3	0.024	0.042	0.044	1.0
		1	0.080	0.14	0.089	1.6
		3	0.24	0.42	0.13	3.2
		10	0.80	1.4	0.20	7.0
PFOS	Seacat et al.[88]	0.03	0.0015	0.0025	0.0040	0.6
		0.13	0.0065	0.011	0.017	0.6
		0.34	0.017	0.029	0.044	0.7
		1.33	0.066	0.11	0.15	0.7
PFBA	Butenhoff et al.[89]	1.2	0.0028	0.0012	0.0061	0.2
		6	0.014	0.0061	0.014	0.4
		30	0.071	0.030	0.052	0.6
PFHxA	Loveless et al.[90]	20	0.0017	0.00032	—	—
		100	0.0083	0.0016	—	—
		500	0.041	0.0081	—	—
PFOA	Perkins et al.[91]	0.06	0.0047	0.0059	0.0071	0.8
		0.64	0.050	0.062	0.041	1.5
		1.94	0.15	0.19	0.070	2.7
		6.5	0.51	0.63	0.14	4.5
PFNA	Mertens et al.[92]	0.025	0.0044	0.010	—	—
		0.125	0.022	0.051	—	—
		0.6	0.11	0.25	—	—
PFDoDA	Kato et al.[93]	0.1	0.0019	0.0049	—	—
		0.5	0.0096	0.024	—	—
		2.5	0.048	0.12	—	—
HFPO-DA	Haas[94]	0.1	0.000051	0.000069	—	—
		10	0.0051	0.00069	—	—
		100	0.051	0.0069	—	—

Note: —, no data; HFPO-DA, hexafluoropropylene oxide-dimer acid; PFBA, perfluorobutanoic acid; PFBS, perfluorobutane sulfonic acid; PFDoDA, perfluorododecanoic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexane sulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid.

models were capable of predicting the serum concentrations for single-dose exposure with ratios between modeled and reported values ranging from 0.8 to 1.2 and the repeated-dose experimental data with ratios between modeled and reported values ranging from 0.2 to 7.0. We subsequently succeeded in modeling internal doses for 10 PFAS in the male rat by using these compartment models. These internal doses were used to calculate nine internal RPFs based on liver effects in the male rat. Because PFAS contribute to common adverse health effects (such as liver effects and immune effects), it is strongly recommended to assess the risk of PFAS as combined, cumulative, exposure.[45,56] In the current study, quantitative internal RPFs are provided for as many PFAS as currently achievable to facilitate a group approach for PFAS risk assessment.

Previously, Gomis et al.[79] employed toxicokinetic modeling to qualitatively rank the potencies of six PFAS based on external dose, serum concentration (AUC), and target organ concentration (AUC). The ranking of PFAS in the current paper from most

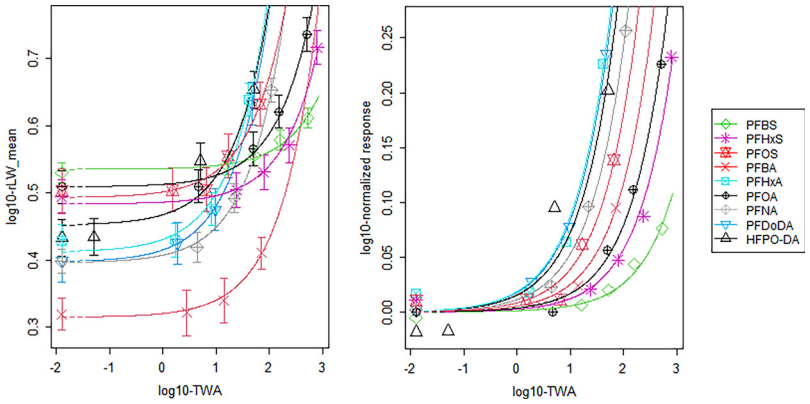

Figure 5. Dose–response curves for mean relative liver weight increase (g/100 g body weight) plotted against the internal dose (expressed as TWA in milligrams per milliliter) on a $\log_{10}$-scale for nine PFAS, obtained by fitting the exponential model with a study-specific (modeled) background response (left plot) and normalized to the background response (right plot). Note: Figures represent geometric mean response for each dose group, as derived from the summary data (Table S1). For clarity, the whiskers indicating the 95% confidence intervals of the means are not plotted in the right plot. HFPO-DA, hexafluoropropylene oxide-dimer acid; PFAS, per- and polyfluoroalkyl substances; PFBA, perfluorobutanoic acid; PFBS, perfluorobutane sulfonic acid; PFDoDA, perfluorododecanoic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexane sulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid; rLW, relative liver weight; TWA, time-weighted average.

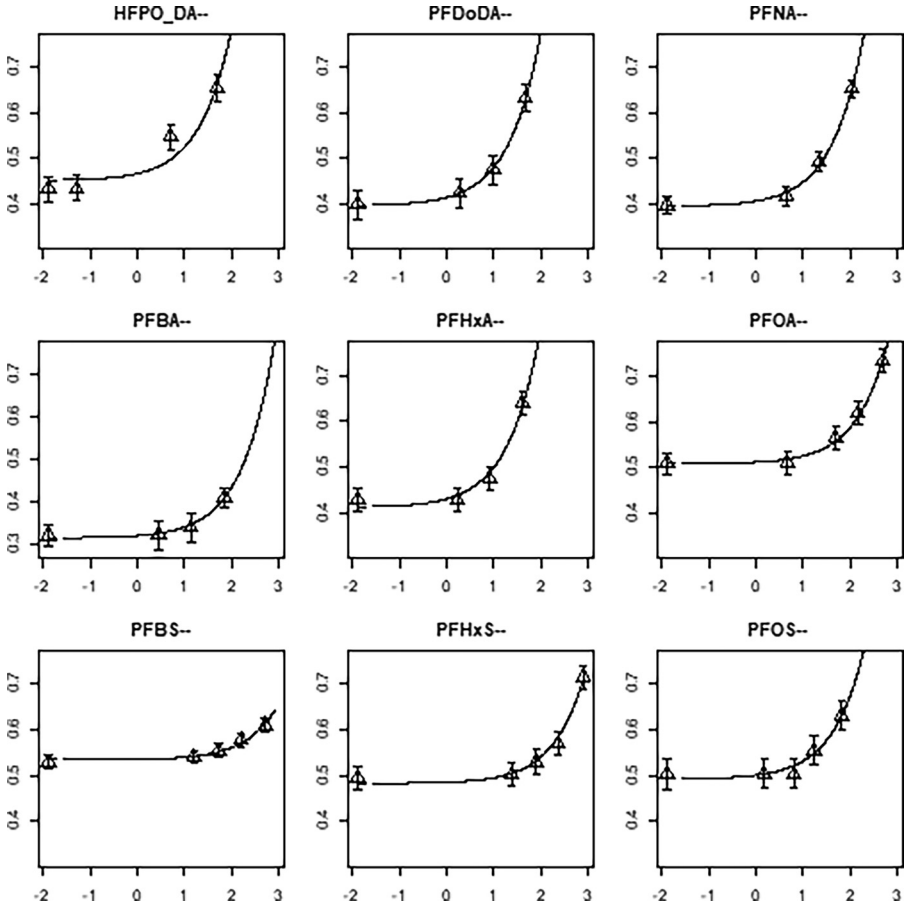

Figure 6. Individual dose–response curves for relative liver weight increase (g/100 g body weight) (*y*-axis) plotted against the internal dose (expressed as TWA in milligrams per milliliter) (*x*-axis) on a $\log_{10}$-scale for nine PFAS, obtained by simultaneously fitting the exponential model to confirm the same curve shape is applicable to all PFAS. Note: Triangles and whiskers are the geometric mean relative liver weights and their 95% confidence intervals, as derived from the summary data reported in Table S1. HFPO-DA, hexafluoropropylene oxide-dimer acid; PFAS, per- and polyfluoroalkyl substances; PFBA, perfluorobutanoic acid; PFBS, perfluorobutane sulfonic acid; PFDoDA, perfluorododecanoic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexane sulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid; TWA, time-weighted average.

potent to least potent is different from that based on serum concentrations in Gomis et al.[79] This difference in approach likely has a methodological foundation. Whereas we both used animal toxicity data and based our analysis on the same end point (i.e., liver toxicity in the male rat), Gomis et al.[79] used a different kinetic model concept to convert from external to internal dose, used AUCs instead of TWAs, and used no adverse observed effect levels (NOAELs) instead of dose–response analysis. Nevertheless, both studies point in the same direction: Relative potencies for PFAS are, overall, more comparable at the blood level than based on the oral external dose, and hence, toxicokinetic differences among PFAS significantly affect potency. Specifically, the range of internal RPFs for the PFAS in our study ranged from 0.2 to 10, whereas the external RPFs for the same PFAS ranged from 0.001 to 10.[77] This observation is also supported by the findings of Vogs et al.,[113] who report that recalculation of external effect concentrations to internal effect concentrations in zebrafish reduced the difference in potency between PFAS considerably.

As a general remark, our analysis did not allow us to take into account the uncertainty of the underlying toxicokinetic data, which is captured by the ratio difference in measured and modeled serum concentrations, and hence this uncertainty is not reflected in the CIs of the RPFs that are reported in Table S5. The largest ratio differences between measured and modeled point estimate serum concentrations at the end of the study ranged from 0.2 to 7.0 (Table 4). Based on the toxicokinetic information currently available for the PFAS that we could derive RPFs for, we are not able to reduce this uncertainty. We used calibrated models based on the best toxicokinetic data currently available: the single-dose experiments that include multiple measurements over time. The repeated-dose studies did not

Table 5. Internal relative potency factors (RPFs) for PFAS based on time-weighted average (TWA) serum concentrations expressed against relative liver weight in the male rat obtained from Subchronic repeated-dose toxicity studies. RPFs were derived by simultaneous fitting of parallel curves and expressing the relative potency of each PFAS against the index compound (PFOA) that received an RPF of 1.

Compound	Internal RPF
PFBS	0.2
PFHxS	0.6
PFOS	3
PFBA	2
PFHxA	10
PFOA	1
PFNA	5
PFDoDA	10
HFPO-DA	9

Note: HFPO-DA, hexafluoropropylene oxide-dimer acid; PFAS, per- and polyfluoroalkyl substances; PFBA, perfluorobutanoic acid; PFBS, perfluorobutane sulfonic acid; PFDoDA, perfluorododecanoic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexane sulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid.

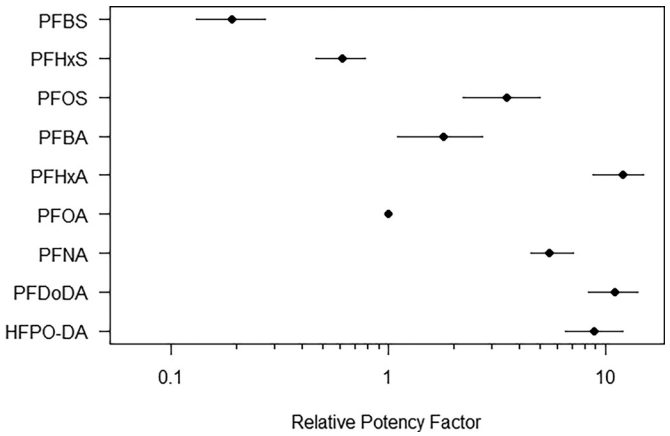

Figure 7. The internal relative potencies of nine PFAS based on relative liver weight (circles), plotted on a log10-scale. The horizontal lines indicate the 90% confidence interval ranges. Perfluorooctanoic acid (PFOA) was used as index compound (i.e., relative potency factor = 1). Note: HFPO-DA, hexafluoropropylene oxide-dimer acid; PFAS, per- and polyfluoroalkyl substances; PFBA, perfluorobutanoic acid; PFBS, perfluorobutane sulfonic acid; PFDoDA, perfluorododecanoic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexane sulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid.

allow for derivation of toxicokinetic model parameters due to absence of information or a too-limited number of measurements taken over time. In that regard, all internal RPFs, but especially the internal RPF for PFDoDA, should be interpreted with caution. For this substance, there was only one high-dose toxicokinetic study available,[114] and the difference with the dosing regimen in the subchronic study was substantial (toxicokinetic study 50 mg/kg body weight/d; top-dose toxicity study 5 mg/kg body weight/d). This circumstance forced us to make more crude assumptions in comparison with the analyses of the other substances (see Supplementary Material, "Toxicokinetic model parameterization") because we were unable to verify the toxicokinetic model for repeated-dose exposure of PFDoDA with empirical data because data were lacking. As a consequence, we have reservations as to whether the model is able to predict the internal concentration at the dosing range of the toxicity study and consequently whether our analysis correctly reflects the internal RPF of PFDoDA.

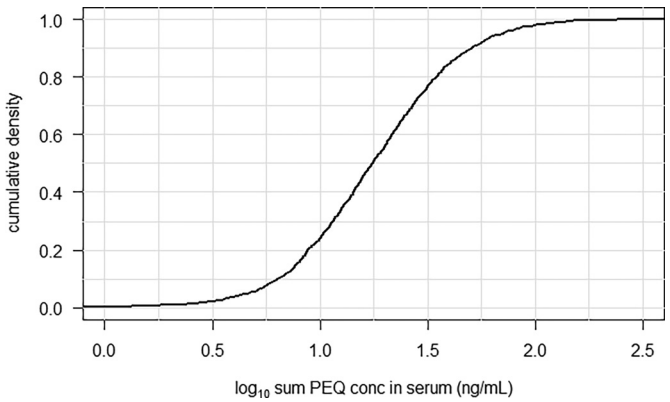

Figure 8. Cumulative density plot of the sum PFOA Equivalent (PEQ) concentration in serum (ng/mL) from the 2017–2018 National Health and Nutrition Examination Survey (NHANES) study population, ages 12 and older ($n = 1{,}929$).[82] Note: The black line represents the sum PEQ of all PFAS [perfluorohexanoic acid (PFHxA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorohexane sulfonic acid (PFHxS), perfluorooctane sulfonic acid (PFOS), hexafluoropropylene oxide-dimer acid (HFPO-DA)] of which internal relative potency factors were derived.

Although the adverse effects resulting from PFAS exposure manifest at the target site, relative potencies based on serum concentrations are required for risk assessment based on human biomonitoring data. This requirement is because exposure to PFAS is usually assessed based on blood measurements in human biomonitoring studies.[76] Also, Health-Based Guidance Values (HBGVs) are available on the level of the serum concentration.[44,45] To estimate the cumulative risk in a study population, the sum PEQ distribution should be compared to such an HBGV expressed on serum concentration level based on toxicity data for PFOA itself[44] or based on toxicity data of the sum of PFAS where PFOA is the main contributor.[45] In using RPFs, it is assumed that the relative toxicities are the same in humans and in male rats. Potential interspecies differences are not corrected for in derivation of the RPFs but in derivation of the HBGV. Thus, when using the RPF methodology, researchers assume that the same interspecies factor for possible remaining differences in toxicokinetics (e.g., serum-to-liver partitioning) and toxicodynamics, based on information of the index compound, is also applicable to the other PFAS.

For some PFAS with relatively short elimination half-lives, single blood samples represent exposure as a snapshot in time, which is surrounded by uncertainty of intake and elimination processes. As our analysis illustrates, the C_{min} and C_{max} values in male rats may differ considerably for substances with a high elimination rate (see Figure 4). To observe potential background exposure of PFAS in humans that is missed by looking at blood solely, paired urine–blood samples could be taken for substances with shorter elimination half-lives[16,45] to verify whether blood concentrations provide a proper estimate of the internal exposure. Moreover, researchers should be aware of compounds that may be metabolized in the body, such as mono-, di-, and tripolyfluoroalkyl phosphate esters (PAPs),[115] fluorotelomer alcohols (FTOHs),[36,115–117] perfluoroalkyl sulfonamidoethanols (FOSEs),[118,119] perfluoroalkyl sulfonamides (FOSAs),[118,119] and perfluoroalkyl phosphinic acids (PFPiA).[120] Here as well, it is important to take account of the exposure metric used. For intake doses, it is most convenient to use the RPF of the parent compound, under the assumption that the metabolization pathway between species is similar. Conversely, at the internal level, it is important to consider the RPFs for the stable intermediate and final metabolites, particularly for those that bioaccumulate. Because the concentrations of PFAA in the human blood are generally high in comparison with their precursors,[115] exposure to these substances may be taken into account at least to some extent.

We acknowledge that the proposed method is subject to further refinement, but we are of the opinion that the actual risk can be estimated more accurately when potency differences between PFAS are taken into account and when several PFAS can be included in the risk calculation,[121] which is in line with the goal to account for cumulative exposure to substances.[122] To further align the RPF methodology with the existing HBGVs for PFAS, it is essential to study the relative potencies for effects other than liver toxicity in the male rat. This applies to the external as well as the internal RPFs. Discussion of this issue was already touched on in Bil et al.,[77] where it was noted that it would be relevant to validate whether RPFs based on data for other end points, sex, species, and life stages would result in comparable estimates of relative potencies and whether liver toxicity RPFs, which will probably be the largest set of *in vivo* RPFs, may be a proxy of the differences in overall toxicity among PFAS. One potential database for further study would be that of developmental toxicity studies with mice.[123–136] Models may also be established for female mice, because toxicokinetic information for model implementation is readily available in the public literature.[64,65,67,68,71,80,137] This approach would allow

modeling of systemic internal exposure and derivation of internal RPFs for developmental toxicity. For human (epidemiological) data sets, it has not been illustrated yet whether it is feasible to obtain a relevant set of internal RPFs. Currently, efforts are being made to investigate whether it is possible to derive internal RPFs based on human data sets for parameters indicative of immune functioning. These analyses will be supplemented by internal RPF analyses for immune-relevant parameters from animal toxicity studies. This development is particularly relevant in the European context, because the point of departure for the EFSA TWI is based on immunosuppression in children.[138]

Due to the absence of internal RPFs, exposure to PFDA, PFUnDA, PFHpS, Me-FOSAA, and 6:2 Cl-PFESA measured in the NHANES study could not be included in the example calculation, which consequently underestimates cumulative exposure and hence underestimates the cumulative risk. Furthermore, it is not unlikely that the general population is exposed to an unknown fraction of PFAS, because an increasing proportion of unknown total organofluorine has been observed in several human biomonitoring studies in recent years.[27,139] Wishing to have a better understanding of the risk associated with cumulative exposure to PFAS, we encourage researchers to focus on identifying the unknown fraction of PFAS in human blood and to subsequently investigate the toxicokinetics and toxicity of these substances. This investigation could be done by performing *in vivo* animal experiments, but researchers may also employ other sources, such as *in vitro* studies and accompanying quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) methods,[140,141] or human epidemiological studies. Such data would allow researchers to extend the number of internal RPFs and to verify the animal toxicity data-derived RPFs with alternative resources.

Although emissions of PFAS in Europe may be expected to diminish due to several policy initiatives, such as the planned restriction for nonessential uses of PFAS under REACH, exposure to the bulk of PFAS already emitted to the environment may continue, due to the persistence of these substances and their tendency to remain in the water phase. Consequently, development of mixture-based risk assessment tools for PFAS will remain an important topic in the future and deserves the necessary attention.

Conclusion

Human biomonitoring is of added value in risk assessment because it provides an empirical measure for aggregate exposure to multiple chemicals and thereby reflects the body burden. Consequently, risk assessment based on such information transcends the compartmentalized regulatory frameworks, specific uses, and isolated exposure routes for which risk assessments are historically performed. In the current study, we present a methodology that enables researchers to assess the risk of exposure to multiple PFAS with primary input from human biomonitoring studies. Internal RPFs were derived based on systemic internal exposure in the male rat resulting in liver effects. PFBA, PFHxA, PFNA, PFDoDA, PFOS, and HFPO-DA were found to be more potent than PFOA at the serum level, whereas PFBS and PFHxS were found to be less potent. This work highlights the importance of using the correct set of RPFs, because potency changes when based on internal systemic exposure in comparison with external exposure. Data availability was a restricting factor in derivation of internal RPFs. To better understand the cumulative risk associated with human exposure to PFAS, it is essential to identify the PFAS in the unknown organofluorine fraction in human blood and to obtain toxicokinetic and toxicity data for more PFAS.

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